APPENDIX G

FISHER'S EXACT TEST

1. Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) is a statistical method based on the hypergeometric probability distribution that can be used to test if the proportion of successes is the same in two Bernoulli (binomial) populations. When used with the *Ceriodaphnia dubia* data, it provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli population. Additionally, since it is a conservative test, a pair-wise comparison error rate of 0.05 is suggested rather that an experiment-wise error rate.

2. The basis for Fisher's Exact Test is a 2×2 contingency table. However, in order to use this table the contingency table must be arranged in the format shown in Table G.1. From the 2×2 table, set up for the control and the concentration you wish to compare, you can determine statistical significance by looking up a value in the table provided later in this section.

TABLE G.1. FORMAT FOR CONTINGENCY TABLE

	Number of		
	Successes	Failures	Number of Observations
Row 1	a	A - a	A
Row 2	b	B - b	B
Total	a + b	[(A + B) - a - b]	A + B

3. Arrange the table so that the total number of observations for row one is greater than or equal to the total for row two ($A \geq B$). Categorize a success such that the proportion of successes for row one is greater than or equal to the proportion of successes for row two ($a/A \geq b/B$). For the *Ceriodaphnia dubia* survival data, a success may be 'alive' or 'dead', whichever causes $a/A \geq b/B$. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is Table G.5. Enter Table G.5 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05 in Table G.5, then the survival proportion for the effluent concentration is significantly different from the survival proportion for the control. A dash or absence of entry in Table G.5 indicates that no contingency table in that class is significant.

4. To illustrate Fisher's Exact Test, a set of survival data (Table G.2) from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test will be used.

5. For each control and effluent concentration construct a 2x2 contingency table.

6. For the control and effluent concentration of 1% the appropriate contingency table for the test is given in Table G.3.

TABLE G.2. EXAMPLE OF FISHER'S EXACT TEST: *CERIODAPHNIA DUBIA* MORTALITY DATA

Effluent Concentration (%)	No. Dead	Total[1]
Control	1	9
1	0	10
3	0	10
6	0	10
12	0	10
25	10	10

[1] Total number of live adults at the beginning of the test.

7. Since 10/10 > 8/9, the category 'alive' is regarded as a success. For A = 10, B = 9 and, a = 10, under the column headed 0.05, the value from Table G.5 is b = 5. Since the value of b (b = 8) from the contingency table (Table G.3), is greater than the value of b (b = 5) from Table G.5, the test concludes that the proportion of survival is not significantly different for the control and 1% effluent.

8. The contingency tables for the combinations of control and effluent concentrations of 3%, 6%, 12% are identical to Table G.3. The conclusion of no significant difference in the proportion of survival for the control and the level of effluent would also remain the same.

9. For the combination of control and 25% effluent, the contingency table would be constructed as Table G.4. The category 'dead' is regarded as a success, since 10/10 > 1/9. The b value (b = 1) from the contingency table (Table G.4) is less than the b value (b = 5) from the table of significance levels of b (Table G.5). Thus, the percent mortality for 25% effluent is significantly greater than the percent mortality for the control. Thus, the NOEC and LOEC for survival are 12% and 25%, respectively.

TABLE G.3. 2×2 CONTINGENCY TABLE FOR CONTROL AND 1% EFFLUENT

	Number of		
	Alive	Dead	Number of Observations
1% Effluent	10	0	10
Control	8	1	9
Total	18	1	19

Table G.4. 2x2 CONTINGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		
	Dead	Alive	Number of Observations
25% Effluent	10	0	10
Control	1	8	9
Total	11	8	19

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1]

	α	0-05	0-025	0-01	0-005
A=3 B=3	3	0 -050			——
A=4 B=4	4	1 -014	1 -014	——	——
3	4	0 -029	——	——	——
A=5 B=5	5	1 -024	1 -024	0 -004	0 -004
	4	0 -024	1 -024	——	——
4	5	1 -048	0 -008	0 -008	——
	4	0 -040	——	——	——
3	5	0 -018	0 -018	——	——
2	5	0 -048	——	——	——
A=6 B=6	6	2 -030	1 -008	1 -008	0 -001
	5	1 -040	0 -008	0 -008	——
	4	0 -030	——	——	——
5	6	1 -015^{+}	0 -015^{+}	0 -002	0 -002
	5	0 -013	0 -013	——	——
	4	0 -045^{+}	——	——	——
4	6	1 -033	0 -005^{-}	0 -005^{-}	0 -005^{-}
	5	0 -024	0 -024	——	——
3	6	0 -012	0 -012	——	——
	5	0 -048	——	——	——
2	6	0 -036	——	——	——
A=7 B=7	7	3 -035^{-}	2 -010^{+}	1 -002	1 -002
	6	1 -015^{-}	1 -015^{-}	0 -002	0 -002
	5	1 -010^{+}	0 -010^{+}	——	——
	4	0 -035-	——	——	——
6	7	2 -021	2 -021	1 -005^{-}	1 -005^{-}
	6	1 -025^{+}	0 -004	0 -004	0 -004
	5	0 -016	0 -016	——	——
	4	0 -049	——	——	——
5	7	2 -045^{+}	1 -010^{+}	0 -001	0 -001
	6	1 -045^{+}	0 -008	0 -008	——
	5	0 -027	——	——	——
	7	1 -024	1 -024	——	0 -003
	6	0 -015^{+}	0 -015^{+}	0 -003	——
	5	0 -045^{+}	——	——	——
	7	0 -008	0 -008	0 -008	——
	6	0 -033	——	——	——
	7	0 -028	——	——	——
A=8	8	4 -038	3 -013	2 -003	2 -003
	7	2 -020	2 -020	1 -005^{+}	0 -001
	6	1 -020	1 -020	0 -003	0 -003
	5	0 -013	0 -013		
	4	0 -038			
7	8	3 -026	2 -007	2 -007	1 -001
	7	2 -035^{-}	1 -009	1 -009	0 -001
	6	1 -032	0 -006	0 -006	
	5	0 -019	0 -019		
6	8	2 -015^{-}	2 -015^{-}	1 -003	1 -003
	7	1 -016	1 -016	0 -002	0 -002
	6	0 -009	0 -009	0 -009	
	5	0 -028	——	——	
5	8	2 -035^{-}	1 -007	1 -007	0 -001
	7	1 -032	0 -005^{-}	0 -005^{-}	0 -005^{-}
	6	0 -016	0 -016	——	
	5	0 -044	——	——	
4	8	1 -018	1 -018	0 -002	0 -002
	7	0 -010^{+}	0 -010^{+}	——	
	6	0 -030	——	——	
3	8	0 -006	0 -006	0 -006	
	7	0 -024	0 -024	——	
2	8	0 -022	0 -022	——	
A=9	9	5 -041	4 -015^{-}	3 -005^{-}	3 -005^{-}
	8	3 -025^{-}	3 -025^{-}	2 -008	1 -002
	7	2 -028	1 -008	1 -008	0 -001
	6	1 -025^{-}	1 -025^{-}	0 -005^{-}	0 -005^{-}
	5	0 -015^{-}	0 -015^{-}	——	
	4	0 -041	——	——	
8	9	4 -029	3 -009	3 -009	2 -002
	8	3 -043	2 -013	1 -003	1 -003
	7	2 -044	1 -012	0 -002	0 -002
	6	1 -036	0 -007	0 -007	
	5	0 -020	0 -020	——	
7	9	3 -019	3 -019	2 -005	2 -005^{-}
	8	2 -024	2 -024	1 -006	0 -001
	7	1 -020	1 -020	0 -003	0 -003
	6	0 -010^{+}	0 -010^{+}	——	
	5	0 -029	——	——	
6	9	3 -044	2 -011	1 -002	1 -002
	8	2 -047	1 -011	0 -001	0 -001
	7	1 -035^{-}	0 -006	0 -006	
	6	0 -017	0 -017	——	
	5	0 -042	——	——	

[1] The table shows:(1) In bold type, for given a, A and B, the value of b ([a) which is just significant at the probability level quoted (one-tailed test); and (2) In small type, for given A, B and r = a + b, the exact probability (if there is independence) that b is equal to or less than the integer shown in bold type. From Pearson and Hartley (1962).

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability 0-05	0-025	0-01	0-005
A=9 B=5	9	2 -027	1 -005^{-}	1 -005^{-}	1 -005^{-}
	8	1 -023	1 -023	0 -003	0 -003
	7	0 -	0 -010^{+}	——	——
	6	0 -028	——	——	——
4	9	1 -014	1 -014	0 -001	0 -001
	8	0 -007	0 -007	0 -007	——
	7	0 -021	0 -021	——	——
	6	0 -049	——	0 -005	——
3	9	1 -	0 -005^{-}	0 -005^{-}	0 -005^{-}
	8	0 -018	0 -018	——	——
	7	0 -	——	——	——
2	9	0 -018	0 -018	——	——
A=10 B=10	10	6 -043	5 -016	4 -005^{+}	3 -002
	9	4 -029	3 -010^{-}	3 -010	2 -003
	8	3 -	2 -012	1 -003	1 -003
	7	2 -	1 -010^{-}	1 -010^{-}	0 -002
	6	1 -029	0 -005^{+}	0 -005^{+}	——
	5	0 -016	0 -016	——	——
	4	0 -043	——	——	——
9	10	5 -033	4 -011	3 -003	3 -003
	9	4 -	3 -017	2 -005^{-}	2 -005^{-}
	8	2 -019	2 -019	1 -004	1 -004
	7	1 -	1 -015^{-}	0 -002	0 -002
	6	1 -040	0 -008	0 -008	——
	5	0 -022	0 -022	——	——
8	10	4 -023	4 -023	3 -007	2 -002
	9	3 -032	2 -009	2 -009	1 -002
	8	2 -031	1 -008	1 -008	0 -001
	7	1 -023	1 -023	0 -004	0 -004
	6	0 -011	0 -011	——	——
	5	0 -029	——	——	——
7	10	3 -	3 -015^{-}	2 -003	2 -003
	9	2 -018	2 -018	1 -004	1 -004
	8	1 -013	1 -013	0 -002	0 -002
	7	1 -036	0 -006	0 -006	——
	6	0 -017	0 -017	——	——
	5	0 -041	——	——	——
6	10	3 -036	2 -008	2 -008	1 -001
	9	2 -036	1 -008	1 -008	0 -001
	8	1 -024	1 -024	0 -003	0 -003
	7	0 -	0 -010^{+}	——	——
	6	0 -026	——	——	——
5	10	2 -022	2 -022	1 -004	1 -004
	9	1 -017	1 -017	0 -002	0 -002
	8	1 -	0 -007	0 -007	——
	7	0 -	0 -019	——	——
	6	0 -	——	——	——

	α	Probability 0-05	0-025	0-01	0-005
A=10 B=4	10	1 -011	1 -011	0 -001	0 -001
	9	1 -041	0 -005^{-}	0 -005^{-}	0 -005^{-}
	8	0 -	0 -015^{-}	——	——
	7	0 -	——	——	——
3	10	1 -038	0 -003	0 -003	0 -003
	9	0 -014	0 -014	——	——
	8	0 -	——	——	——
2	10	0 -	0 -015^{+}	——	——
	9	0 -	——	——	——
A=11 B=11	11	7 -	6 -018	5 -006	4 -002
	10	5 -032	4 -012	3 -004	3 -004
	9	4 -040	3 -015^{-}	2 -004	2 -004
	8	3 -043	2 -015^{-}	1 -004	1 -004
	7	2 -040	1 -012	0 -002	0 -002
	6	1 -032	0 -006	0 -006	——
	5	0 -018	0 -018	——	——
	4	0 -	——	——	——
10	11	6 -	5 -012	4 -004	4 -004
	10	4 -021	4 -021	3 -007	2 -002
	9	3 -024	3 -024	2 -007	1 -002
	8	2 -023	2 -023	1 -006	0 -001
	7	1 -017	1 -017	0 -003	0 -003
	6	1 -043	0 -009	0 -009	——
	5	0 -023	0 -023	——	——
9	11	5 -026	4 -008	4 -008	3 -002
	10	4 -038	3 -012	2 -003	2 -003
	9	3 -040	2 -012	1 -003	1 -003
	8	2 -	1 -009	1 -009	0 -001
	7	1 -	1 -025^{-}	0 -004	0 -004
	6	0 -012	0 -012	——	——
	5	0 -030	——	——	——
8	11	4 -018	4 -018	3 -005^{-}	3 -005^{-}
	10	3 -024	3 -024	2 -006	1 -001
	9	2 -022	2 -022	1 -005^{-}	1 -005^{-}
	8	1 -	1 -015^{-}	0 -002	0 -002
	7	1 -037	0 -007	0 -007	——
	6	0 -017	0 -017	——	——
	5	0 -040	——	——	——
7	11	4 -043	3 -011	2 -002	2 -002
	10	3 -047	2 -013	1 002	1 -002
	9	2 -039	1 -009	1 -009	0 -001
	8	1 -	1 -025^{-}	0 -004	0 -004
	7	0 -	0 -010^{+}	——	——
	6	0 -	0 -025^{-}	——	——
6	11	3 -	2 006	2 -006	1 -001
	10	2 -	1 -005^{+}	1 -005^{+}	0 -001
	9	1 -	1 -018	0 -002	0 -002

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability 0-05	0-025	0-01	0-005
A=11 B=6	8	1 .043	0 .007	0 .007	——
	7	0 .017	0 .017	——	——
	6	0 .037	——	——	——
5	11	2 .018	2 .018	1 .003	1 .003
	10	1 .013	1 .013	0 .001	0 .001
	9	1 .036	0 .005-	0 .005-	0 .005-
	8	0 .013	0 .013	——	——
	7	0 .029	——	——	——
4	11	1 .009	1 .009	1 .009	0 .001
	10	1 .033	0 .004	0 .004	0 .004
	9	0 .011	0 .011	——	——
	8	0 .026	——	——	——
3	11	1 .033	0 .003	0 .003	0 .003
	10	0 .011	0 .011	——	——
	9	0 .027	——	——	——
2	11	0 .013	0 .013	——	——
	10	0 .038	——	——	——
A=12 B=12	12	8 .047	7 .019	6 .007	5 .002
	11	6 .034	5 .014	4 .005-	4 .005-
	10	5 .045-	4 .018	3 .006	2 .002
	9	4 .050-	3 .020	2 .006	1 .001
	8	3 .050-	2 .018	1 .005-	1 .005-
	7	2 .045-	1 .014	0 .002	0 .002
	6	1 .034	0 .007	0 .007	——
	5	0 .019	0 .019	——	——
	4	0 .047	——	——	——
11	12	7 .037	6 .014	5 .005-	5 .005-
	11	5 .024	5 .024	4 .008	3 .002
	10	4 .029	3 .010+	2 .003	2 .003
	9	3 .030	2 .009	2 .009	1 .002
	8	2 .026	1 .007	1 .007	0 .001
	7	1 .019	1 .019	0 .003	0 .003
	6	1 .045-	0 .009	0 .009	——
	5	0 .024	0 .024	——	——
10	12	6 .029	5 .010	5 .010-	4 .003
	11	5 .043	4 .015+	3 .005-	3 .005-
	10	4 .048	3 .017	2 .005-	2 .005-
	9	3 .046	2 .015-	1 .004	1 .004
	8	2 .038	1 .010+	0 .002	0 .002
	7	1 .026	0 .005-	0 .005-	0 .005-
	6	0 .012	0 .012	——	——
	5	0 .030	——	——	——
9	12	5 .021	5 .021	4 .006	3 .002
	11	4 .029	3 .009	3 .009	2 .002
	10	3 .029	2 .008	2 .008	1 .002
	9	2 .024	2 .024	1 .006	0 .001
	8	1 .016	1 .016	0 .002	0 .002

	α	Probability 0-05	0-025	0-01	0-005
A=12 B=9	7	1 .037	0 .007	0 .007	——
	6	0 .017	0 .017	——	——
	5	0 .039	——	——	——
8	12	5 .049	4 .014	3 .004	3 .004
	11	3 .018	3 .018	2 .004	2 .004
	10	2 .015+	2 .015+	1 .003	1 .003
	9	2 .040	1 .010-	1 .010-	0 .001
	8	1 .025-	1 .025-	0 .004	0 .004
	7	0 .010+	0 .010+	——	——
	6	0 .024	0 .024	——	——
7	12	4 .036	3 .009	3 .009	2 .002
	11	3 .038	2 .010-	2 .010-	1 .002
	10	2 .029	1 .006	1 .006	0 .001
	9	1 .017	1 .017	0 .002	0 .002
	8	1 .040	0 .007	0 .007	——
	7	0 .016	0 .016	——	——
	6	0 .034	——	——	——
6	12	3 .025-	3 .025-	2 .005-	2 .005-
	11	2 .022	2 .022	1 .004	1 .004
	10	1 .013	1 .013	0 .002	0 .002
	9	1 .032	0 .005-	0 .005-	0 .005-
	8	0 .011	0 .011	——	——
	7	0 .025-	0 .025-	——	——
	6	0 .050-	——	——	——
5	12	2 .015-	2 .015	1 .002	1 .002
	11	1 .010-	1 .010-	1 .010-	0 .001
	10	1 .028	0 .003	0 .003	0 .003
	9	0 .009	0 .009	0 .009	——
	8	0 .020	0 .020	——	——
	7	0 .041	——	——	——
4	12	2 .050	1 .007	1 .007	0 .001
	11	1 .027	0 .003	0 .003	0 .003
	10	0 .008	0 .008	0 .008	——
	9	0 .019	0 .019	——	——
	8	0 .038	——	——	——
3	12	1 .029	0 .002	0 .002	0 .002
	11	0 .009	0 .009	0 .009	——
	10	0 .022	0 .022	——	——
	9	0 .044	——	——	——
2	12	0 .011	0 .011	——	——
	11	0 .033	——	——	——
A=13 B=13	13	9 .048	8 .020	7 .007	6 .003
	12	7 .037	6 .015+	5 .006	4 .002
	11	6 .048	5 .021	4 .008	3 .002
	10	4 .024	4 .024	3 .008	2 .002
	9	3 .024	3 .024	2 .008	1 .002
	8	2 .021	2 .021	1 .006	0 .001

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability			
		0-05	0-025	0-01	0-005
A=13 B=13	7	2 -048	1 -015+	0 -003	0 -003
	6	1 -037	0 -007	0 -007	——
	5	0 -020	0 -020	——	——
	4	0 -048		——	——
12	1	8 -039	7 -015-	6 -005+	5 -002
	1	6 -027	5 -010-	5 -010-	4 -003
	1	5 -033	4 -013	3 -004	3 -004
	1	4 -036	3 -013	2 -004	2 -004
	9	3 -034	2 -011	1 -003	1 -003
	8	2 -029	1 -008	1 -008	0 -001
	7	1 -020	1 -020	0 -004	0 -004
	6	1 -046	0 -010-	0 -010-	——
	5	0 -024	0 -024	——	——
11	1	7 -031	6 -011	5 -003	5 -003
	1	6 -048	5 -018	4 -006	3 -002
	1	4 -021	4 -021	3 -007	2 -002
	1	3 -021	3 -021	2 -006	1 -001
	9	3 -050-	2 -017	1 -004	1 -004
	8	2 -040	1 -011	0 -002	0 -002
	7	1 -027	0 -005-	0 -005-	0 -005-
	6	0 -013	0 -013	——	——
		0 -030		——	——
10	1	6 -024	6 -024	5 -007	4 -002
	1	5 -035-	4 -012	3 -003	3 -003
	1	4 -037	3 -012	2 -003	2 -003
	1	3 -033	2 -010+	1 -002	1 -002
	9	2 -026	1 -006	1 -006	0 -001
	8	1 -017	1 -017	0 -003	0 -003
	7	1 -038	0 -007	0 -007	——
	6	0 -017	0 -017	——	——
	5	0 -038		——	——
9	1	5 -017	5 -017	4 -005-	4 -005-
	1	4 -023	4 -023	3 -007	2 -001
	1	3 -022	3 -022	2 -006	1 -001
	1	2 -017	2 -017	1 -004	1 -004
	9	2 -040	1 -010+	0 -001	0 -001
	8	1 -025-	1 -025-	0 -004	0 -004
	7	0'-010+	0 -010+	——	——
	6	0 -023	0 -023	——	——
	5	0 -049		——	——
8	1	5 -042	4 -012	3 -003	3 -003
	1	4 -047	3 -014	2 -003	2 -003
	1	3 -041	2 -011	1 -002	1 -002
	1	2 -029	1 -007	1 -007	0 -001
	9	1 -017	1 -017	0 -002	0 -002
	8	1 -037	0 -006	0 -006	——
	7	0 -015-	0 -015-	——	——
	6	0 -032		——	——
7	1	4 -031	3 -007	3 -007	2 -001
	1	3 -031	2 -007	2 -007	1 -001

	α	Probability			
		0-05	0-025	0-01	0-005
A=13 B=7	11	2 -022	2 -022	1 -004	1 -004
	10	1 -012	1 -012	0 -002	0 -002
	9	1 -029	0 -004	0 -004	0 -004
	8	0 -010+	0 -010+	——	——
	7	0 -022	0 -022	——	——
	6	0 -044	——	——	——
6	13	3 -021	3 -021	2 -004	2 -004
	12	2 -017	2 -017	1 -003	1 -003
	11	2 -046	1 -010-	1 -010-	0 -001
	10	1 -024	1 -024	0 -003	0 -003
	9	1 -050-	0 -008	0 -008	——
	8	0 -017	0 -017	——	——
	7	0 -034	——	——	——
5	13	2 -012	2 -012	1 -002	1 -002
	12	2 -044	1 -008	1 -008	0 -001
	11	1 -022	1 -022	0 -002	0 -002
	10	1 -047	0 -007	0 -007	——
	9	0 -015-	0 -015-	——	——
	8	0 -029	——	——	——
4	13	2 -044	1 -006	1 -006	0 -000
	12	1 -022	1 -022	0 -002	0 -002
	11	0 -006	0 -006	0 -006	——
	10	0 -015-	0 -015-	——	——
	9	0 -029	——	——	——
3	13	1 -025	1 -025	0 -002	0 -002
	12	0 -007	0 -007	0 -007	——
	11	0 -018	0 -018	——	——
	10	0 -036	——	——	——
2	13	0 -010-	0 -010-	0 -010-	——
	12	0 -029	——	——	——
A=14	14	10 -049	9 -020	8 -008	7 -003
	13	8 -038	7 -016	6 -006	5 -002
	12	6 -023	6 -023	5 -009	4 -003
	11	5 -027	4 -011	3 -004	3 -004
	10	4 -028	3 -011	2 -003	2 -003
	9	3 -027	2 -009	2 -009	1 -002
	8	2 -023	2 -023	1 -006	0 -001
	7	1 -016	1 -016	0 -003	0 -003
	6	1 -038	0 -008	0 -008	——
	5	0 -020	0 -020	——	——
	4	0 -049	——	——	——
13	14	9 -041	8 -016	7 -006	6 -002
	13	7 -029	6 -011	5 -004	5 -004
	12	6 -037	5 -015+	4 -005+	3 -002
	11	5 -041	4 -017	3 -006	2 -001
	10	4 -041	3 -016	2 -005-	2 -005-
	9	3 -038	2 -013	1 -003	1 -003
	8	2 -031	1 -009	1 -009	0 -001

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=14	7	1 -021	1 -021	0 -004	0 -004	A=14 B=7	14	4 -026	3 -006	3 -006	2 -001
	6	1 -048	0 -010+	——	——		13	3 -025	2 -006	2 -006	1 -001
	5	0 -025-	0 -025-	——	——		12	2 -017	2 -017	1 -003	1 -003
12	1	8 -033	7 -012	6 -004	6 -004		11	2 -041	1 -009	1 -009	0 -001
	1	6 -021	6 -021	5 -007	4 -002		10	1 -021	1 -021	0 -003	0 -003
	1	5 -025+	4 -009	4 -009	3 -003		9	1 -043	0 -007	0 -007	——
	1	4 -026	3 -009	3 -009	2 -002		8	0 -015-	0 -015-	——	——
	1	3 -024	3 -024	2 -007	1 -002		7	0 -030	——	——	——
	9	2 -019	2 -019	1 -005-	1 -005-	6	14	3 -018	3 -018	2 -003	2 -003
	8	2 -042	1 -012	0 -002	0 -002		13	2 -014	2 -014	1 -002	1 -002
	7	1 -028	0 -005+	0 -005+	——		12	2 -037	1 -007	1 -007	0 -001
	6	0 -013	0 -013	——	——		11	1 -018	1 -018	0 -002	0 -002
	5	0 -030	——	——	——		10	1 -038	0 -005+	0 -005+	——
11	1	7 -026	6 -009	6 -009	5 -003		9	0 -012	0 -012	——	——
	1	6 -039	5 -014	4 -004	4 -004		8	0 -024	0 -024	——	——
	1	5 -043	4 -016	3 -005-	3 -005-		7	0 -044	——	——	——
	1	4 -042	3 -015-	2 -004	2 -004	5	14	2 -010+	2 -010+	1 -001	1 -001
	1	3 -036	2 -011	1 -003	1 -003		13	2 -037	1 -006	1 -006	0 -001
	9	2 -027	1 -007	1 -007	0 -001		12	1 -017	1 -017	0 -002	0 -002
	8	1 -017	1 -017	0 -003	0 -003		11	1 -038	0 -005-	0 -005-	0 -005-
	7	1 -038	0 -007	0 -007	——		10	0 -011	0 -011	——	——
	6	0 -017	0 -017	——	——		9	0 -022	0 -022	——	——
	5	0 -038	——	——	——		8	0 -040	——	——	——
10	1	6 -020	6 -020	5 -006	4 -002	4	14	2 -039	1 -005-	1 -005-	1 -005-
	1	5 -028	4 -009	4 -009	3 -002		13	1 -019	1 -019	0 -002	0 -002
	1	4 -028	3 -009	3 -009	2 -002		12	1 -044	0 -005-	0 -005-	0 -005-
	1	3 -024	3 -024	2 -007	1 -001		11	0 -011	0 -011	——	——
	1	2 -018	2 -018	1 -004	1 -004		10	0 -023	0 -023	——	——
	9	2 -040	1 -011	0 -002	0 -002		9	0 -041	——	——	——
	8	1 -024	1 -024	0 -004	0 -004	3	14	1 -022	1 -022	0 -001	0 -001
	7	0 -010-	0 -010-	0 -010-	——		13	0 -006	0 -006	0 -006	——
	6	0 -022	0 -022	——	——		12	0 -015-	0 -015-	——	——
	5	0 -047	——	——	——		11	0 -029	——	——	——
9	1	6 -047	5 -014	4 -004	4 -004	2	14	0 -008	0 -008	0 -008	——
	1	4 -018	4 -018	3 -005-	3 -005-		13	0 -025	0 -025	——	——
	1	3 -017	3 -017	2 -004	2 -004		12	0 -050	——	——	——
	1	3 -042	2 -012	1 -002	1 -002						
	1	2 -029	1 -007	1 -007	0 -001						
	9	1 -017	1 -017	0 -002	0 -002						
	8	1 -036	0 -006	0 -006	——	A=15 B=15	15	11 -050-	10 -021	9 -008	8 -003
	7	0 -014	0 -014	——	——		14	9 -040	8 -018	7 -007	6 -003
	6	0 -030	——	——	——		13	7 -025+	6 -010+	5 -004	5 -004
8	1	5 -036	4 -010-	4 -010-	3 -002		12	6 -030	5 -013	4 -005-	4 -005-
	1	4 -039	3 -011	2 -002	2 -002		11	5 -033	4 -013	3 -005-	3 -005-
	1	3 -032	2 -008	2 -008	1 -001		10	4 -033	3 -013	2 -004	2 -004
	1	2 -022	2 -022	1 -005-	1 -005-		9	3 -030	2 -010+	1 -003	1 -003
	1	2 -048	1 -012	0 -002	0 -002		8	2 -025+	1 -007	1 -007	0 -001
	9	1 -026	0 -004	0 -004	0 -004		7	1 -018	1 -018	0 -003	0 -003
	8	0 -009	0 -009	0 -009	——		6	1 -040	0 -008	0 -008	——
	7	0 -020	0 -020	——	——		5	0 -021	0 -012	——	——
	6	0 -040	——	——	——		4	0 -050-	——	——	——

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=15 B=14	15	10 -042	9 -017	8 -006	7 -002	A=15 B=9	13	4 -042	3 -013	2 -003	2 -003
	14	8 -031	7 -013	6 -005-	6 -005-		12	3 -032	2 -009	2 -009	1 -002
	13	7 -041	6 -017	5 -007	4 -002		11	2 -021	2 -021	1 -005-	1 -005-
	12	6 -046	5 -020	4 -007	3 -002		10	2 -045-	1 -011	0 -002	0 -002
	11	5 -048	4 -020	3 -007	2 -002		9	1 -024	1 -024	0 -004	0 -004
	10	4 -046	3 -018	2 -006	1 -001		8	1 -048	0 -009	0 -009	——
	9	3 -041	2 -014	1 -004	1 -004		7	0 -019	0 -019	——	——
	8	2 -033	1 -009	1 -009	0 -001		6	0 -037	——	——	——
	7	1 -022	1 -022	0 -004	0 -004	8	15	5 -032	4 -008	4 -008	3 -002
	6	1 -049	0 -011	——	——		14	4 -033	3 -009	3 -009	2 -002
	5	0 -025+	——	——	——		13	3 -026	2 -006	2 -006	1 -001
13	15	9 -035-	8 -013	7 -005-	7 -005-		12	2 -017	2 -017	1 -003	1 -003
	14	7 -023	7 -023	6 -009	5 -003		11	2 -037	1 -008	1 -008	0 -001
	13	6 -029	5 -011	4 -004	4 -004		10	1 -019	1 -019	0 -003	0 -003
	12	5 -031	4 -012	3 -004	3 -004		9	1 -038	0 -006	0 -006	——
	11	4 -030	3 -011	2 -003	2 -003		8	0 -013	0 -013	——	——
	10	3 -026	2 -008	2 -008	1 -002		7	0 -026	——	——	——
	9	2 -020	2 -020	1 -005+	0 -001		6	0 -050-	——	——	——
	8	2 -043	1 -013	0 -002	0 -002	7	15	4 -023	4 -023	3 -005-	3 -005-
	7	1 -029	0 -005+	0 -005+	——		14	3 -021	3 -021	2 -004	2 -004
	6	0 -013	0 -013	——	——		13	2 -014	2 -014	1 -002	1 -002
	5	0 -031	——	——	——		12	2 -032	1 -007	1 -007	0 -001
12	15	8 -028	7 -010-	7 -010-	6 -003		11	1 -015+	1 -015+	0 -002	0 -002
	14	7 -043	6 -016	5 -006	4 -002		10	1 -032	0 -005-	0 -005-	0 -005-
	13	6 -049	5 -019	4 -007	3 -002		9	0 -010+	0 -010+	——	——
	12	5 -049	4 -019	3 -006	2 -002		8	0 -020	0 -020	——	——
	11	4 -045+	3 -017	2 -005-	2 -005-		7	0 -038	——	——	——
	10	3 -038	2 -012	1 -003	1 -003	6	15	3 -015+	3 -015+	2 -003	2 -003
	9	2 -028	1 -007	1 -007	0 -001		14	2 -011	2 -011	1 -002	1 -002
	8	1 -018	1 -018	0 -003	0 -003		13	2 -031	1 -006	1 -006	0 -001
	7	1 -038	0 -007	0 -007	——		12	1 -014	1 -014	0 -002	0 -002
	6	0 -017	0 -017	——	——		11	1 -029	0 -004	0 -004	0 -004
	5	0 -037	——	——	——		10	0 -009	0 -009	0 -009	——
11	15	7 -022	7 -022	6 -007	5 -002		9	0 -017	0 -017	——	——
	14	6 -032	5 -011	4 -003	4 -003		8	0 -032	——	——	——
	13	5 -034	4 -012	3 -003	3 -003	5	15	2 -009	2 -009	2 -009	1 -001
	12	4 -032	3 -010+	2 -003	2 -003		14	2 -032	1 -005-	1 -005-	1 -005-
	11	3 -026	2 -008	2 -008	1 002		13	1 -014	1 -014	0 -001	0 -001
	10	2 -019	2 -019	1 -004	1 -004		12	1 -031	0 -004	0 -004	0 -004
	9	2 -040	1 -011	0 -002	0 -002		11	0 -008	0 -008	0 -008	——
	8	1 -024	1 -024	0 -004	0 -004		10	0 -016	0 -016	——	——
	7	1 -049	0 -010-	0 -010-	——		9	0 -030	——	——	——
	6	0 -022	0 -022	——	——	4	15	2 -035+	1 -004	1 -004	1 -004
	5	0 -046	——	——	——		14	1 -016	1 -016	0 -001	0 -001
10	15	6 -017	6 -017	5 -005-	5 -005-		13	1 -037	0 -004	0 -004	0 -004
	14	5 -023	5 -023	4 -007	3 -002		12	0 -009	0 -009	0 -009	——
	13	4 -022	4 -022	3 -007	2 -001		11	0 -018	0 -018	——	——
	12	3 -018	3 -018	2 -005-	2 -005-		10	0 -033	——	——	——
	11	3 -042	2 -013	1 -003	1 -003	3	15	1 -020	1 -020	0 -001	0 -001
	10	2 -029	1 -007	1 -007	0 -001		14	0 -005-	0 -005-	0 -005-	0 -005-
	9	1 -016	0 -016	0 -002	0 -002		13	0 -012	0 -012	——	——
	8	1 -034	0 -006	0 -006	——		12	0 -025-	0 -025-	——	——
	7	0 -013	1 -013	——	——		11	0 -043	——	——	——
	6	0 -028	——	——	——	2	15	0 -007	0 -007	0 -007	——
9	15	6 -042	5 -012	4 -003	4 -003		14	0 -022	0 -022	——	——
	14	5 -047	4 -015-	3 -004	3 -004		13	0 -044	——	——	——

APPENDIX H

SINGLE CONCENTRATION TOXICITY TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR RECEIVING WATER

1. To statistically compare a control with one concentration, such as 100% effluent or the instream waste concentration, a t-test is the recommended analysis. The t-test is based on the assumptions that the observations are independent and normally distributed and that the variances of the observations are equal between the two groups.

2. Shapiro Wilk's test may be used to test the normality assumption (see Appendix B for details). If the data do not meet the normality assumption, the nonparametric test, Wilcoxon's Rank Sum Test, may be used to analyze the data. An example of this test is given in Appendix F. Since a control and one concentration are being compared, the K = 1 section of Table F.5 contains the needed critical values.

3. The F test for equality of variances is used to test the homogeneity of variance assumption. When conducting the F test, the alternative hypothesis of interest is that the variances are not equal.

4. To make the two-tailed F test at the 0.01 level of significance, put the larger of the two variances in the numerator of F.

$$F = \frac{S_1^2}{S_2^2} \quad where\ S_1^2 > S_2^2$$

5. Compare F with the 0.005 level of a tabled F value with $n_1 - 1$ and $n_2 - 1$ degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups.

6. A set of *Ceriodaphnia dubia* reproduction data from an effluent screening test will be used to illustrate the F test. The raw data, mean and variance for the control and 100% effluent are given in Table H.1.

TABLE H.1. *CERIODAPHNIA DUBIA* REPRODUCTION DATA FROM AN EFFLUENT SCREENING TEST

	Replicate											
	1	2	3	4	5	6	7	8	9	10	$\bar{X}$	S^2
Control	36	38	35	35	28	41	37	33	.	.	35.4	14.5
100% Effluent	23	14	21	7	12	17	23	8	18	.	15.9	36.6

7. Since the variability of the 100% effluent is greater than the variability of the control, S^2 for the 100% effluent concentration is placed in the numerator of the F statistic and S^2 for the control is placed in the denominator.

$$F = \frac{36.61}{14.55}$$

8. There are 9 replicates for the effluent concentration and 8 replicates for the control. Thus, the numerator degrees of freedom is 8 and the denominator degrees of freedom is 7. For a two-tailed test at the 0.01 level of

significance, the critical F value is obtained from a table of the F distribution (Snedecor and Cochran, 1980). The critical F value for this test is 8.68. Since 2.52 is not greater than 8.68, the conclusion is that the variances of the control and 100% effluent are homogeneous.

9. **EQUAL VARIANCE T-TEST**

9.1 To perform the t-test, calculate the following test statistic:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where: $\bar{Y}_1$ = Mean for the control

$\bar{Y}_2$ = Mean for the effluent concentration

$$S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

9.2 Since we are usually concerned with a decreased response from the control, such as a decrease in survival or a decrease in reproduction, a one-tailed test is appropriate. Thus, compare the calculated t with a critical t, where the critical t is at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the calculated t exceeds the critical t, the mean responses are declared different.

9.3 Using the data from Table H.1 to illustrate the t-test, the calculation of t is as follows:

$$t = \frac{35.4 - 15.9}{5.13\sqrt{\frac{1}{8} + \frac{1}{9}}} = 7.82$$

Where:

$$S_p = \sqrt{\frac{(8-1)14.5 + (9-1)36.6}{(8+9-2)}} = 5.13$$

9.4 For an 0.05 level of significance test with 15 degrees of freedom the critical t is 1.754 (Note: Table D.5 for K = 1 includes the critical t values for comparing two groups). Since 7.82 is greater than 1.754, the conclusion is that the reproduction in the 100% effluent concentration is significantly lower than the control reproduction.

10. **UNEQUAL VARIANCE T-TEST**

10.1 If the F test for equality of variance fails, the t-test is still a valid test. However, the denominator of the t statistic is adjusted as follows:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where: $\bar{Y}_1$ = Mean for the control

$\bar{Y}_2$ = Mean for the effluent concentration

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

10.2 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df' = \frac{(n_1 - 1)\,(n_2 - 1)}{(n_2 - 1)\,C^2 + (1 - C)^2 (n_1 - 1)}$$

Where:

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

10.3 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

10.4 The t-test is then conducted as the equal variance t-test. The calculated t is compared to the critical t at the 0.05 significance level with the modified degrees of freedom. If the calculated t exceeds the critical t, the mean responses are found to be statistically different.

APPENDIX I

PROBIT ANALYSIS

1. This program calculates the EC1 and EC50 (or LC1 and LC50), and the associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled, executable version of the program can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.1 Data input is illustrated by a set of total mortality data (Figure I.1) from a fathead minnow embryo-larval survival and teratogenicity test. The program requests the following input:

1. Desired output of abbreviated (A) or full (F) output? (Note: only abbreviated output is shown below.)
2. Output designation (P = printer, D = disk file).
3. Title for the output.
4. The number of exposure concentrations.
5. Toxicant concentration data.

2.2 The program output for the abbreviated output includes the following:

1. A table of the observed proportion responding and the proportion responding adjusted for the controls (see Figure I.2).
2. The calculated chi-square statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
3. Estimated LC1 and LC50 values and associated 95% confidence intervals (see Figure I.2).

USEPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Do you wish abbreviated (A) or full (F) input/output? A
Output to printer (P) or disk file (D)? P
Title ? Example of Probit Analysis

Number responding in the control group = ? 2
Number of animals exposed in the concurrent control group = ? 20
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 0.5
Number responding = ? 2
Number exposed = ? 20

Concentration = ? 1.0
Number responding = ? 1
Number exposed = ? 20

Concentration = ? 2.0
Number responding = ? 4
Number exposed = ? 20

Concentration = ? 4.0
Number responding = ? 16
Number exposed = ? 20

Concentration = ? 8.0
Number responding = ? 20
Number exposed = ? 20

Number	Number Conc.	Number Resp.	Exposed
1	0.5000	2	20
2	1.0000	1	20
3	2.0000	4	20
4	4.0000	16	20
5	8.0000	20	20

Do you wish to modify your data ? N

The number of control animals which responded = 2
The number of control animals exposed = 20
Do you wish to modify these values ? N

Figure I.1. Sample Data Input for USEPA Probit Analysis program, Version 1.5.

Example of Probit Analysis

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	20	2	0.1000	0.0000
0.5000	20	2	0.1000	0.0174
1.0000	20	1	0.0500	-.0372
2.0000	20	4	0.2000	0.1265
4.0000	20	16	0.8000	0.7816
8.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.441
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

Example of Probit Analysis

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC 1.00	1.346	0.453	1.922
LC/EC 50.00	3.018	2.268	3.672

Figure I.2. USEPA Probit Analysis Program Used for Calculating LC/EC Values, Version 1.5.

APPENDIX J

SPEARMAN-KARBER METHOD

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber Method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

4. To calculate the LC50 using the Spearman-Karber Method, the following must be true: 1) the smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero, and 2) the smoothed adjusted proportion mortality for the highest effluent concentration must be one.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

6. The Spearman-Karber Method is illustrated below using a set of mortality data from a Fathead Minnow Larval Survival and Growth test. These data are listed in Table J.1.

TABLE J.1. EXAMPLE OF SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25%	2	0.05
12.5%	0	0.00
25.0%	0	0.00
50.0%	26	0.65
100.0%	40	1.00

7. Let $p_0, p_1, ..., p_k$ denote the observed response proportion mortalities for the control and k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^{s} = p_{i}^{s} = \frac{(p_i + p_{i-1})}{2}$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

7.1 For the data in this example, because the observed mortality proportions for the control and the 6.25% effluent concentration are greater than the observed response proportions for the 12.5% and 25.0% effluent concentrations, the responses for these four groups must be averaged:

$$p_0^s = p_1^s = p_2^s = p_3^s = \frac{0.05+0.05+0.00+0.00}{4} = \frac{0.10}{4} = 0.025$$

7.2 Since $p_4 = 0.65$ is larger than p_3^s, set $p_4^s = 0.65$. Similarly, $p_5 = 1.00$ is larger than p_4^s, so set $p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table J.2.

8. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: p_i^a = $(p_i^s - p_0^s) / (1 - p_0^s)$

p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = p_3^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.025 - 0.025}{1 - 0.025} = \frac{0.0}{0.975} = 0.0$$

$$p_4^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{0.650 - 0.025}{1 - 0.025} = \frac{0.0625}{0.975} = 0.641$$

$$p_5^a = \frac{p_5^s - p_0^s}{1 - p_0^s} = \frac{1.000 - 0.025}{1 - 0.025} = \frac{0.975}{0.975} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table J.2. A plot of the smoothed, adjusted data is shown in Figure J.1.

9. Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^a)(x_i + x_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control.

9.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

$$
\begin{aligned}
m = \; & [(0.000 - 0.000)\,(0.7959 + 1.0969)]/2 \; + \\
& [(0.000 - 0.000)\,(1.0969 + 1.3979)]/2 \; + \\
& [(0.641 - 0.000)\,(1.3979 + 1.6990)]/2 \; + \\
& [(1.000 - 0.641)\,(1.6990 + 2.0000)]/2 \\
= \; & 1.656527
\end{aligned}
$$

TABLE J.2. EXAMPLE OF SPEARMAN-KARBER METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.025	0.000
6.25%	0.05	0.025	0.000
12.5%	0.00	0.025	0.000
25.0%	0.00	0.025	0.000
50.0%	0.65	0.650	0.641
100.0%	1.00	1.000	1.000

10. Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^{a}(1-p_i^{a})(X_{i+1}+X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control.

10.1 For this example, the estimated variance of m, V(m), is calculated as follows:

$$
\begin{aligned}
V(m) = \; & (0.000)(1.000)(1.3979 - 0.7959)^2/4(39) \; + \\
& (0.000)(1.000)(1.6990 - 1.0969)^2/4(39) \; + \\
& (0.641)(0.359)(2.0000 - 1.3979)^2/4(39) \\
= \; & 0.00053477
\end{aligned}
$$

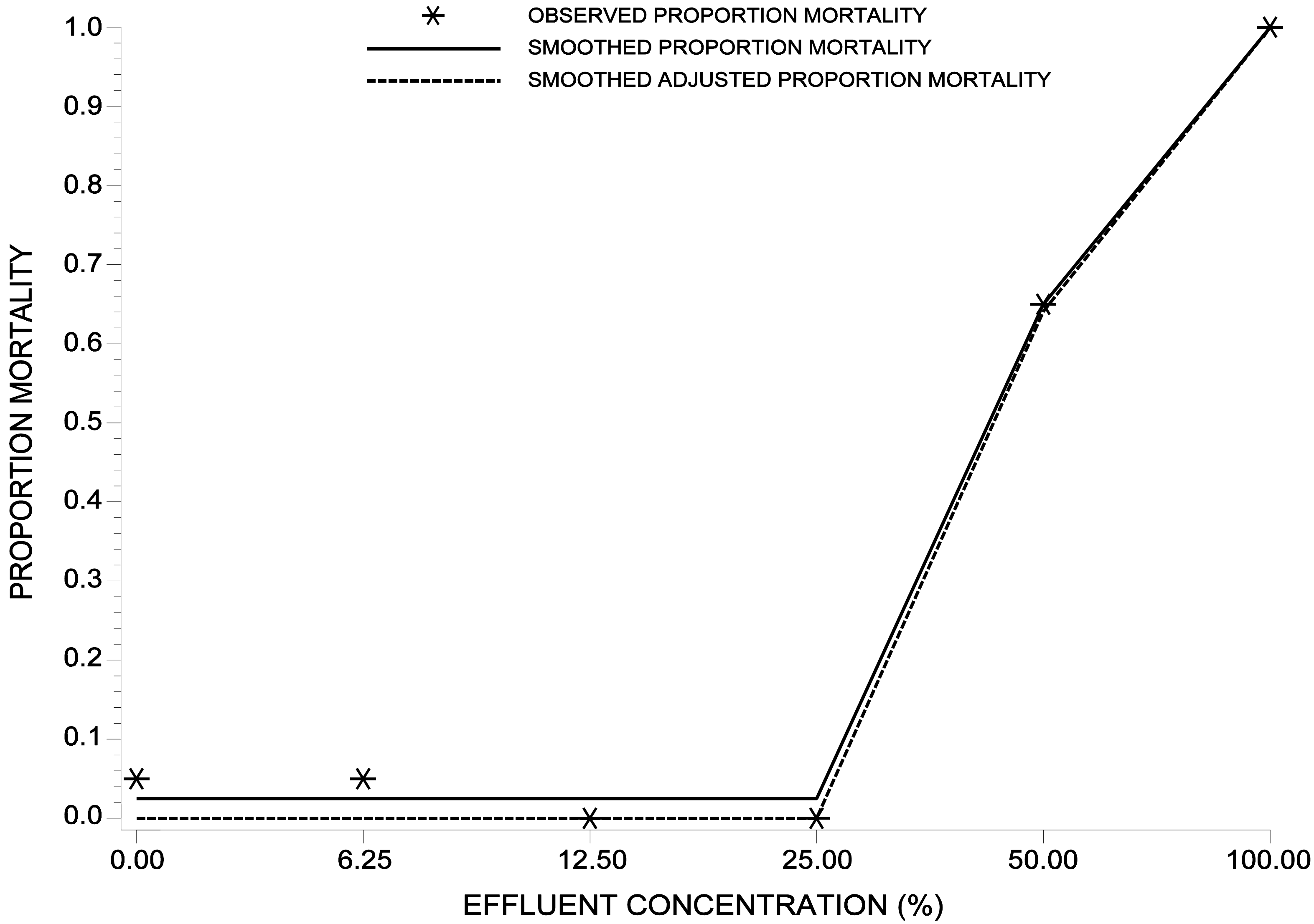

Figure J.1. Plot of the smoothed, adjusted data for the fathead minnow larval survival and growth test.

11. Calculate the 95% confidence interval for m: $m \pm 2.0\sqrt{V(m)}$

11.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.656527 \pm 2\sqrt{0.00053477} = (1.610277,\ 1.702777)$$

12. The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking base_{10} antilogs of the above values.

12.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.656527) = 45.3%.

12.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.610277) = 40.8%

upper limit: antilog(1.702777) = 50.4%

APPENDIX K

TRIMMED SPEARMAN-KARBER METHOD

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber Method, a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton et al, 1977). Appendix The Trimmed Spearman-Karber Method estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Trimmed Spearman-Karber Analysis is recommended only when the requirements for the Probit Method and the Spearman-Karber Method are not met.

4. To calculate the LC50 using the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

6. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \leq p_1 \leq ... \leq p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \leq p_1 \leq ... \leq p_k$, with their average. For example, if p_i is less than p_{i-1} then:

Where: $p^s_{i-1} = p^s_i = (p_i + p_{i-1})/2$

p^s_i = the smoothed observed proportion mortality for effluent concentration i.

7. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: $p^a_i = (p^s_i - p^s_0) / (1 - p^s_0)$

p^s_0 = the smoothed observed proportion mortality for the control

p^s_i = the smoothed observed proportion mortality for effluent concentration i.

8. Calculate the amount of trim to use in the estimation of the LC50 as follows:

Where: $\text{Trim} = \max(p^a_1, 1-p^a_k)$

p^a_1 = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control

p^a_k = the smoothed, adjusted proportion mortality for the highest effluent concentration

k = the number of effluent concentrations, exclusive of the control.

The minimum trim should be calculated for each data set rather than using a fixed amount of trim for each data set.

9. Due to the intensive nature of the calculation for the estimated LC50 and the calculation of the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

10. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method, can be obtained from EMSL-Cincinnati by sending a written request to EMSL, 3411 Church Street, Cincinnati, OH 45244.

11. The Trimmed Spearman-Karber program automatically performs the following functions:

a. Smoothing.
b. Adjustment for mortality in the control.
c. Calculation of the necessary trim.
d. Calculation of the LC50.
e. Calculation of the associated 95% confidence interval.

12. To illustrate the Trimmed Spearman-Karber method using the Trimmed Spearman-Karber computer program, a set of data from a Fathead Minnow Larval Survival and Growth test will be used. The data are listed in Table K.1.

TABLE K.1. EXAMPLE OF TRIMMED SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	2	0.05
25.0	0	0.00
50.0	0	0.00
100.0	32	0.80

12.1 The program requests the following input (Figure K.1):

a. Output destination (D = disk file, P = printer).
b. Control data.
c. Data for each toxicant concentration.

12.2 The program output includes the following (Figure K.2):

a. A table of the concentrations tested, number of organisms exposed, and mortalities.
b. The amount of trim used in the calculation.
c. The estimated LC50 and the associated 95% confidence interval.

```
A:>spearman

 TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5

ENTER DATE OF TEST:
1
ENTER TEST NUMBER:
2
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
 L
ENTER TEST SPECIES NAME:
Fathead minnow
ENTER TOXICANT  NAME:
Effluent
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT:
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
 40
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
2
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL;  MAX = 10):
5
ENTER THE  5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25  12.5  25  50  100
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:        40

ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Days
ENTER DURATION OF TEST:
7
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:        0 2 0 0 32

WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
 y
```

Figure K.1. Example input for Trimmed Spearman-Karber Method.

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1   TEST NUMBER: 2   DURATION: 7 Days
TOXICANT: effluent
SPECIES: fathead minnow

RAW DATA: Concentration (%)	Number Exposed	Mortalities
.00	40	2
6.25	40	0
12.50	40	2
25.00	40	0
50.00	40	0
100.00	40	32

SPEARMAN-KARBER TRIM: 20.41%

SPEARMAN-KARBER ESTIMATES: LC50:   77.28
95% CONFIDENCE LIMITS
ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure K.2. Example output for Trimmed Spearman-Karber Method.

APPENDIX L

GRAPHICAL METHOD

1. The Graphical Method is used to calculate the LC50. It is a mathematical procedure which estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm ($\log_{10}$) of percent effluent concentration. This method does not provide a confidence interval for the LC50 estimate and its use is only recommended when there are no partial mortalities. The only requirement for the Graphical Method is that the observed percent mortalities bracket 50%.

2. For an analysis using the Graphical Method the data must first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps.

3. The Graphical Method is illustrated below using a set of mortality data from an Fathead Minnow Larval Survival and Growth test. These data are listed in Table L.1.

TABLE L.1. EXAMPLE OF GRAPHICAL METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	0	0.00
25.0	0	0.00
50.0	40	1.00
100.0	40	1.00

4. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \leq p_1 \leq ... \leq p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \leq p_1 \leq ... \leq p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^s = p_i^s = (p_i + p_{i-1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

4.1 For the data in this example, because the observed mortality proportions for the 6.25%, 12.5%, and 25.0% effluent concentrations are less than the observed response proportion for the control, the values for these four groups must be averaged:

$$p_0^s = p_1^s = p_2^s = p_3^s = \frac{0.05 + 0.00 + 0.00 + 0.00}{4} = \frac{0.05}{4} = 0.0125$$

4.2 Since $p_4 = p_5 = 1.00$ are larger then 0.0125, set $p_4^s = p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table L.2.

TABLE L.2. EXAMPLE OF GRAPHICAL METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.0125	0.00
6.25	0.00	0.0125	0.00
12.5	0.00	0.0125	0.00
25.0	0.00	0.0125	0.00
50.0	1.00	1.0000	1.00
100.0	1.00	1.0000	1.00

5. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

5.1 Because the smoothed observed proportion mortality for the control group is greater than zero, the responses must be adjusted using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = p_3^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.0125 - 0.0125}{1 - 0.0125} = \frac{0.0}{0.9875} = 0.0$$

$$p_4^a = p_5^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{1.00 - 0.0125}{1 - 0.0125} = \frac{0.9875}{0.9875} = 1.00$$

A table of the smoothed, adjusted response proportions for the effluent concentrations are shown in Table L.2.

5.2 Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the y axis) used for percent effluent concentration and the linear axis (the x axis) used for observed percent mortality. A plot of the smoothed, adjusted data is shown in Figure L.1.

6. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

7. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

7.1 For this example, the two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

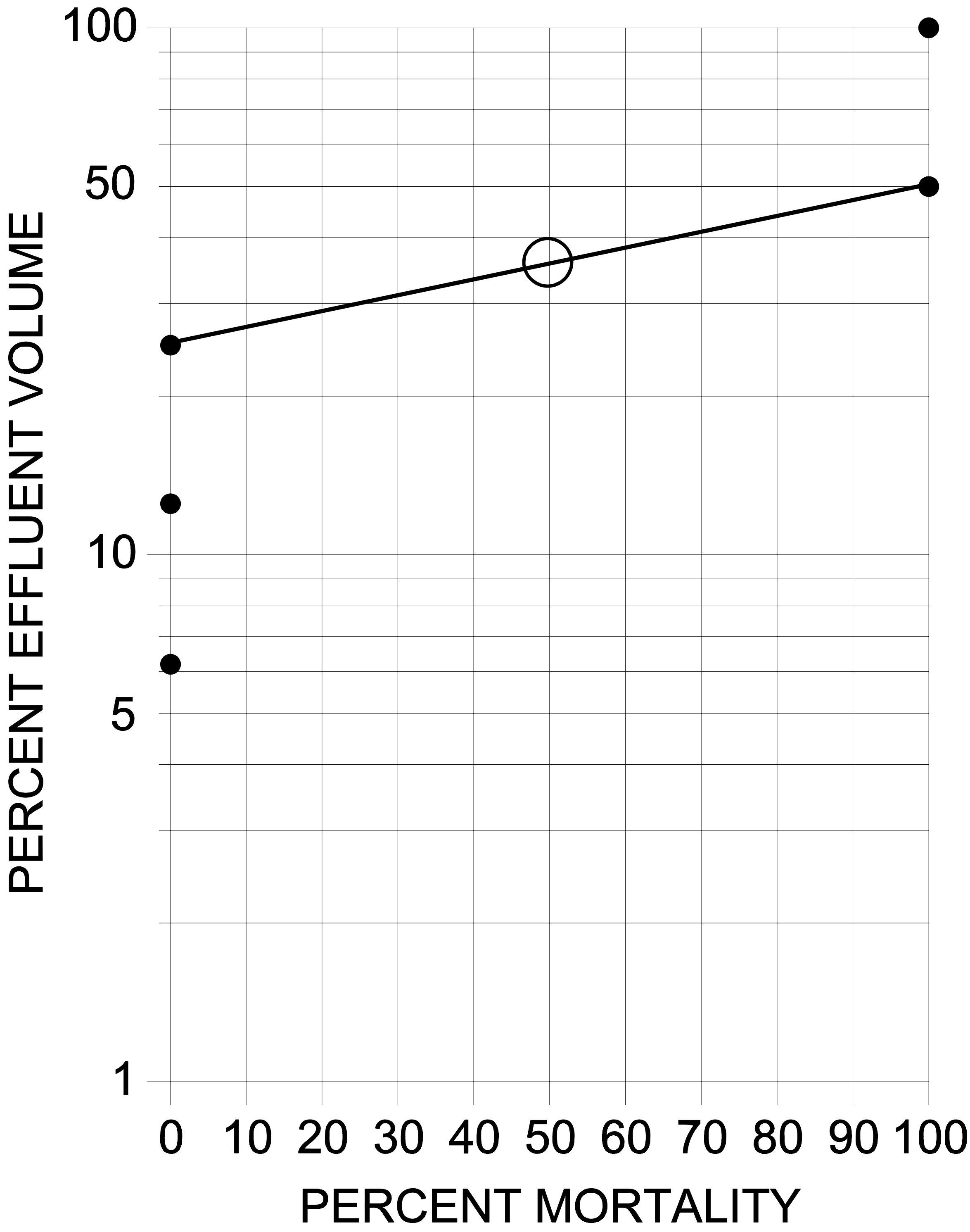

Figure L.1 Plot of the smoothed adjusted response proportions for fathead minnow, *Pimephales promelas*, survival data.

APPENDIX M

LINEAR INTERPOLATION METHOD

1. **GENERAL PROCEDURE**

1.1 The Linear Interpolation Method is used to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms (Inhibition Concentration, or IC). The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

1.2 The Linear Interpolation Method assumes that the responses (1) are monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically nonincreasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. Also, no assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

2. **DATA SUMMARY AND PLOTS**

2.1 Calculate the mean responses for the control and each toxicant concentration, construct a summary table, and plot the data.

3. **MONOTONICITY**

3.1 If the assumption of monotonicity of test results is met, the observed response means ($\bar{Y}_i$) should stay the same or decrease as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means.

3.2 Observed means at each concentration are considered in order of increasing concentration, starting with the control mean ($\bar{Y}_1$). If the mean observed response at the lowest toxicant concentration ($\bar{Y}_2$) is equal to or smaller than the control mean ($\bar{Y}_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration ($\bar{Y}_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. A numerical example of smoothing the data is provided below. (Note: Unusual patterns in the deviations from monotonicity may require an additional step of smoothing). Where $\bar{Y}_i$ decrease monotonically, the $\bar{Y}_i$ become M_i without smoothing.

4. **LINEAR INTERPOLATION METHOD**

4.1 The method assumes a linear response from one concentration to the next. Thus, the ICp is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

4.2 To obtain the estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp = C_J + [\, M_1 \; (1 - p/100) - M_J \,] \; \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

Where: C_J = tested concentration whose observed mean response is greater than $M_1(1 - p/100)$.

C_{J+1} = tested concentration whose observed mean response is less than $M_1(1 - p/100)$.

M_1 = smoothed mean response for the control.

M_J = smoothed mean response for concentration J.

M_{J+1} = smoothed mean response for concentration J + 1.

p = percent reduction in response relative to the control response.

ICp = estimated concentration at which there is a percent reduction from the smoothed mean control response. The ICp is reported for the test, together with the 95% confidence interval calculated by the ICPIN.EXE program described below.

4.3 If the C_J is the highest concentration tested, the ICp would be specified as *greater than* C_J. If the response at the lowest concentration tested is used to extrapolate the ICp value, the ICp should be expressed as a *less than the lowest test concentration.*

5. **CONFIDENCE INTERVALS**

5.1 Due to the use of a linear interpolation technique to calculate an estimate of the ICp, standard statistical methods for calculating confidence intervals are not applicable for the ICp. This limitation is avoided by use a technique known as the bootstrap method as proposed by Efron (1982) for deriving point estimates and confidence intervals.

5.2 In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. The bootstrap method is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ji} is randomly resampled with replacement to produce a new set of data Y_{ji}*, that is statistically equivalent to the original data, but a new and slightly different estimate of the ICp (ICp*) is obtained. This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associate ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 time, the empirical 2.5% and the 97.5% confidence limits are approximately the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988).

5.3 The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

5.4 The bootstrapping method of calculating confidence intervals is computationally intensive. For this reason, all of the calculations associated with determining the confidence intervals for the ICp estimate have been incorporated into a computer program. Computations are most easily done with a computer program such as the revision of the

BOOTSTRP program (USEPA, 1988; USEPA, 1989) which is now called "ICPIN" which is described below in subsection 7.

6. **MANUAL CALCULATIONS**

6.1 DATA SUMMARY AND PLOTS

6.1.1 The data used in this example are the *Ceriodaphnia dubia* reproduction data used in the example in Section 13. Table M.1 includes the raw data and the mean reproduction for each concentration. Data are included for all animals tested regardless of death of the organism. If an animal died during the test without producing young, a zero is entered. If death occurred after producing young, the number of young produced prior to death is entered. A plot of the data is provided in Figure M.1.

TABLE M.1. *CERIODAPHNIA DUBIA* REPRODUCTION DATA

		Effluent Concentration (%)				
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	27	32	39	27	10	0
2	30	35	30	34	13	0
3	29	32	33	36	7	0
4	31	26	33	34	7	0
5	16	18	36	31	7	0
6	15	29	33	27	10	0
7	18	27	33	33	10	0
8	17	16	27	31	16	0
9	14	35	38	33	12	0
10	27	13	44	31	2	0
Mean ($\overline{Y}_i$)	22.4	26.3	34.6	31.7	9.4	0
i	1	2	3	4	5	6

6.2 MONOTONICITY

6.2.1 As can be seen from the plot in Figure M.1, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

6.2.2 Starting with the control mean $\overline{Y}_1 = 22.4$ and $\overline{Y}_2 = 26.3$, we see that $\overline{Y}_1 < \overline{Y}_2$. Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1 + \overline{Y}_2)/2 = 24.35$$

6.2.3 Since $\overline{Y}_3 = 34.6$ is larger than M_2, average $\overline{Y}_3$ with the previous concentrations:

6.2.4 Additionally, $\overline{Y}_4 = 31.7$ is larger than M_3, and is pooled with the first three means. Thus,

$$M_1 = M_2 = M_3 = M_4 = (M_1 + M_2 + M_3 + \overline{Y}_4)/4 = 28.7$$

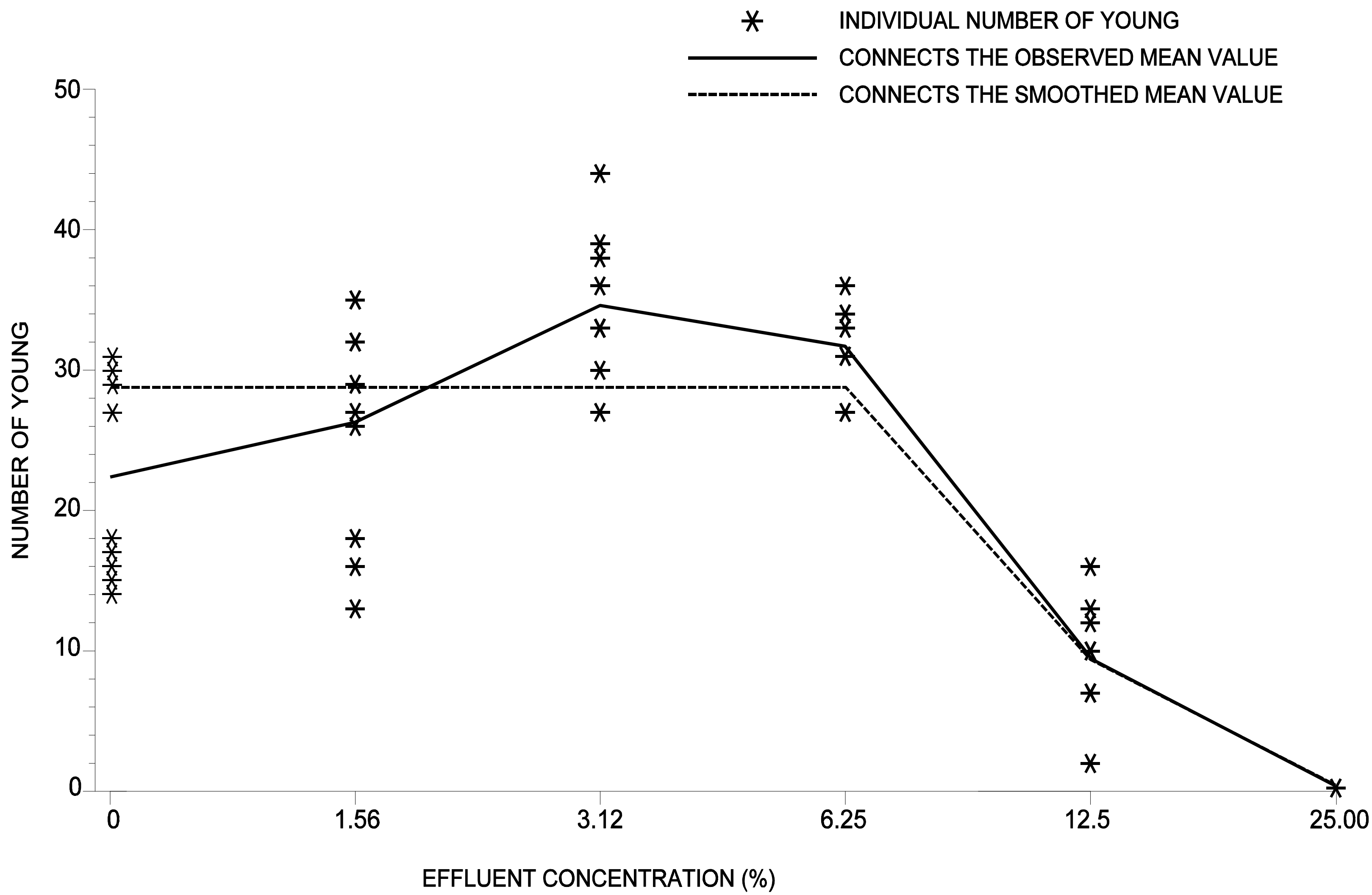

Figure M.1. Plot of raw data, observed means, and smoothed means for the daphnid, *Ceriodaphnia dubia*, reproductive data.

TABLE M.2. *CERIODAPHNIA DUBIA* REPRODUCTION MEAN RESPONSE AFTER SMOOTHING

Effluent Concentration %	i	Response Mean (Y_i) (Young/female)	Smoothed Mean (M_i) (Young/female)
Control	1	22.4	28.75
1.56	2	26.3	28.75
3.12	3	34.6	28.75
6.25	4	31.7	28.75
12.5	5	9.4	9.40
25.0	6	0.0	0.00

6.2.5 Since $M_4 > \overline{Y}_5 = 9.4$, set $M_5 = 9.4$. Likewise, $M_5 > \overline{Y}_6 = 0$ and M_6 becomes 0. Table M.2 contains the smoothed means and Figure M.1 gives a plot of the smoothed response curve.

6.3 LINEAR INTERPOLATION

6.3.1 Estimates of the IC25 and IC50 are calculated using the Linear Interpolation Method. A 25% reduction in reproduction, compared to the controls, would result in a mean reproduction of 21.56 young per adult, where $M_1(1\text{-}p/100) = 28.75(1\text{-}25/100)$. A 50% reduction in reproduction, compared to the controls, would result in a mean reproduction of 14.38 young per adult, where $M_1(1\text{-}p/100) = 28.75(1\text{-}50/100)$. Examining the smoothed means and their associated concentrations (Table M.2), the two effluent concentrations bracketing the reproduction of 21.56 young per adult are C_4 = 6.25% effluent and C_5 = 12.5% effluent. The two effluent concentrations bracketing a response of 14.38 young per adult are also C_4 = 6.25% effluent and C_5 = 12.5% effluent.

6.3.2 Using Equation 1 from 4.2, the estimate of the IC25 is calculated as follows:

$$ICp = C_J + [\, M_1 \,(1 - p/100) - M_J \,] \,\frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC25 = 6.25 + [28.75\,(1 - 25/100) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 8.57% effluent

6.3.3 Using the equation from section 4.2, the estimate of the IC50 is calculated as follows:

$$ICp = C_J + [\, M_1 \,(1 - p/100) - M_J \,] \,\frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC50 = 6.25 + [28.75\,(1 - 50/100) - 28.75]\,\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 10.89% effluent

6.4 CONFIDENCE INTERVALS

6.4.1 Confidence intervals for the ICp are derived using the bootstrap method. As described above, this method involves randomly resampling the individual observations and recalculating the ICp at least 80 times, and determining the mean ICp, standard deviation, and empirical 95% confidence intervals. For this reason, the confidence intervals are calculated using a computer program called ICPIN. This program is described below and is available to carry out all the calculations of both the interpolation estimate (ICp) and the confidence intervals.

7. COMPUTER CALCULATIONS

7.1 The computer program, ICPIN, prepared for the Linear Interpolation Method was written in TURBO PASCAL for IBM compatible PCs. The program (version 2.0) has been modified by Computer Science Corporation, Duluth, MN with funding provided by the Environmental Research Laboratory, Duluth, MN (Norberg-King, 1993). The program was originally developed by Battelle Laboratories, Columbus, OH through a government contract supported by the Environmental Research Laboratory, Duluth, MN (USEPA, 1988). To obtain the program and supporting documentation, send a written request to EMSL-Cincinnati at 3411 Church Street, Cincinnati, OH 45244.

7.2 The ICPIN.EXE program performs the following functions: 1) it calculates the observed response means (Y_i) (response means); 2) it calculates the standard deviations; 3) checks the responses for monotonicity; 4) calculates smoothed means (M_i) (pooled response means) if necessary; 5) uses the means, M_i, to calculate the initial ICp of choice by linear interpolation; 6) performs a user-specified number of bootstrap resamples between 80 and 1000 (as multiples of 40); 7) calculates the mean and standard deviation of the bootstrapped ICp estimates; and 8) provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven (Norberg-King, 1993).

7.3 For the IC*p* calculation, up to twelve treatments can be used (which includes the control). There can be up to 40 replicates per concentration, and the program does not require an equal number of replicates per concentration. The value of p can range from 1% to 99%.

7.4 DATA INPUT

7.4.1 Data is entered directly into the program onscreen. A sample data entry screen in shown in Figure M.2. The program documentation provides guidance on the entering and analysis of data for the Linear Interpolation Method (Norberg-King, 1993).

7.4.2 The user selects the ICp estimate desired (e.g., IC25 or IC50) and the number of resamples to be taken for the bootstrap method of calculating the confidence intervals. The program has the capability of performing any number of resamples from 80 to 1000 as multiples of 40. However, Marcus and Holtzman (1988) recommend a minimum of 80 resamples for the bootstrap method be used and at least 250 resamples are better (Norberg-King, 1993).

7.5 DATA OUTPUT.

7.5.1 The program output includes the following (Figures M.3 and M.4):

1. A table of the concentration identification, the concentration tested and raw data response for each replicate and concentration.
2. A table of test concentrations, number of replicates, concentration (units), response means ($\overline{Y}_i$), standard deviations for each response mean, and the pooled response means (smoothed means; M_i).
3. The linear interpolation estimate of the ICp using the means (M_i). *Use this value for the ICp estimate.*
4. The mean ICp and standard deviation from the bootstrap resampling.
5. The confidence intervals calculated by the bootstrap method for the ICp. Provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is

over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven.

7.6 ICPIN program output for the analysis of the *Ceriodaphnia dubia* reproduction data in Table M.1 is provided in Figures M.3 and M.4.

7.6.1 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.57% effluent. The empirical 95% confidence intervals for the true mean were 8.30% to 8.85% effluent.

7.6.2 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 10.89% effluent. The empirical 95% confidence intervals for the true mean were 10.36% to 11.62% effluent.

ICp Data Entry/Edit Screen Current File:

Conc. ID	1	2	3	4	5	6
Conc. Tested						
Conc. Tested						
Response 1						
Response 2						
Response 3						
Response 4						
Response 5						
Response 6						
Response 7						
Response 8						
Response 9						
Response 10						
Response 11						
Response 12						
Response 13						
Response 14						
Response 15						
Response 16						
Response 17						
Response 18						
Response 19						
Response 20						

F10 for Command Menu Use arrow Keys to Switch Fields

Figure M.2. ICp data entry/edit screen. Twelve concentrating identifications can be used. Data for concentrations are entered in columns 1 through 6. For concentrations 7 through 12 and responses 21-40 the data is entered in additional fields of the same screen.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

```
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: app M   Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration:            7-d
DATA FILE: cerioman.icp
OUTPUT FILE: cerioman.i25
```

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

```
The Linear Interpolation Estimate:     8.5715   Entered P Value: 25

Number of Resamplings:   80
The Bootstrap Estimates Mean:   8.6014 Standard Deviation:     0.1467
Original Confidence Limits:   Lower:     8.3040 Upper:     8.8496
Resampling time in Seconds:     2.53  Random Seed: -1652543090
```

Figure M.3. Example of ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

```
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: app M   Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration:            7-d
DATA FILE: cerioman.icp
OUTPUT FILE: cerioman.i50
```

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

```
The Linear Interpolation Estimate:    10.8931   Entered P Value: 50
```

```
Number of Resamplings:   80
The Bootstrap Estimates Mean:  10.9108 Standard Deviation:     0.3267
Original Confidence Limits:   Lower:    10.3618 Upper:    11.6201
Resampling time in Seconds:     2.58  Random Seed: 340510286
```

Figure M.4. Example of ICPIN program output for the IC50.

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